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Abrogation of G2/M arrest sensitizes curcumin-resistant hepatoma cells to apoptosis

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Abstract In this study, we showed that curcumin treatment resulted in activation of Chk1-mediated G2 checkpoint, which was associated with the induction of G2/M arrest and the resistance of cancer cells to curcumin-induced apoptosis. Further investigation revealed that inhibition of Chk1 significantly abrogated G2/M arrest and sensitized curcumin-resistant cells to apoptosis via upregulation of Bad and in turn the loss of mitochondrial membrane potential. These results indicate that Chk1-mediated G2/M arrest may serve as a mechanism for curcumin resistance and Chk1 represents a potential target for the reversal of this resistance. Our findings should be helpful for clinical application of curcumin.

Keywords: Curcumin; G2/M arrest; Apoptosis; Drug resistance; Hepatoma cells

1. Introduction

Curcumin, a naturally occurring yellow pigment isolated from turmeric, is a well known antioxidant. Increasing evidences also suggest curcumin as a DNA-damaging agent [1–4]. Curcumin is able to induce apoptosis in various cancer cell lines [5] and to suppress cancer formation [6–8], angiogenesis [9] and metastasis [10] in mouse models. Both phase I and II clinical trials show that curcumin is well tolerated and is effective for cancer patients [11,12], suggesting curcumin as a potential drug in cancer prevention and therapy.

Every anticancer drug encounters the problem of drug resistance, which is responsible for limited effect of chemotherapy and high cancer mortality. Therefore, understanding the molecular mechanisms of drug resistance and developing the agents to reverse the resistance are pivotal for improving the prognosis of cancer patients. Most anticancer drugs exert their function via induction of apoptosis. However, these drugs may also activate cell cycle checkpoints, which results in cell cycle arrest and thus protects cells from apoptosis [13,14]. Because malignant cells often have defects in G1 checkpoint, anticancer treatment usually activates G2 checkpoint in tumor cells. Hence, G2 checkpoint in fact represents a survival mechanism for cancer cells to escape apoptosis induced by anticancer therapy [15].

To date, comprehensive studies have been conducted to characterize the anticancer effect of curcumin, but little is known about curcumin resistance. It has been found that curcumin can induce cell cycle arrest at the G2/M phase [16–20], but whether G2/M arrest is associated with the resistance of cancer cells to curcumin-induced cytotoxicity is still unknown. In this study, we aimed to investigate the role of G2/M arrest in curcumin resistance and to identify the potential targets for reversing this resistance. Our findings should be helpful for the clinical application of curcumin.

2. Materials and methods

2.1. Reagents

Rabbit polyclonal antibodies against phospho-Ser317 of Chk1, Chk1, phospho-Thr68 of Chk2, Chk2, phospho-Tyr15 of Cdk1, Bcl-2 (Cell Signaling Technology, Beverly, MA) and Mcl-1 (Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal antibodies against phospho-Thr180/Tyr182 of p38, p38α, Cdk1, Bad (BD Pharmingen International, San Diego, CA), β-actin and Bcl-XL (Santa Cruz); lipofectamine–RNA interference (RNAi) MAX, Trizol, and goat-anti-mouse Alexa 488 (Invitrogen, Carlsbad, CA); p38 inhibitor SB203580 (Promega, Madison, WI). Curcumin (Sigma–Aldrich, St. Louis, MO) was dissolved in DMSO in a concentration of 25 mM and stored at −20 °C in aliquot.

2.2. Apoptosis assay

The hepatoma cell lines, including Huh7, Hep3B, HepG2, SK-Hep-1 and QGY-7703, and colon cancer cell line HCT-116($p53^{-/-}$) were grown in DMEM supplemented with 10% FBS and 2 mM/l L-glutamine. Apoptosis was evaluated by staining with 4′-6′-diamidino-2-phenylindole (Sigma). Cells with nuclear fragmentation or DNA condensation were counted as apoptotic cells. At least 500 cells were counted for each sample.

2.3. Analysis for cell cycle distribution

Cells were resuspended in Krishan's reagent (0.05 mg/ml propidium iodide (PI), 0.1% Na citrate, 0.02 mg/ml ribonuclease A, 0.3% NP-40), incubated at 37 °C for 30 min and applied to fluorescence-activated cell sorting (Becton Dickinson FACS Vantage SE, San Jose, CA).

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Abbreviations: siRNA, short-interfering RNA; RNAi, RNA interference; RT-PCR, reverse transcription-polymerase chain reaction; hPBGD, human porphobilinogen deaminase; FoxM1, forkhead box M1; PI, propidium iodide; p-Chk1, phosphorylated-Ser317-Chk1; p-Chk2, phosphorylated-Thr68-Chk2; p-p38, phosphorylated-Thr180/Tyr182-p38; p-Cdk1, phosphorylated-Tyr15-Cdk1

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2.4. Western blotting

Proteins were separated on 8–15% SDS–polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes, and detected by specific antibodies and ECL kit (Pierce, Rockford, IL). The quantification of protein was performed by densitometry analysis using Glyko Bandscan software (Glyko Inc., USA).

2.5. Transfection of short-interfering RNA (siRNA)

The siRNA sequences targeted human *Chk1* (GeneBank accession no. NM_001274), *Chk2* (GeneBank accession no. NM_007194) and *Bad* (GeneBank accession no. NM_004322) transcripts were designated as si-Chk1, si-Chk2 and si-Bad, respectively. The target sequences selected for RNAi were: 5′-AAG AAG CAG TCG CAG TGA AGA TTG TAG for *Chk1* gene, 5′-AAG AAC CTG AGG ACC AAG AAC CTG AGG for *Chk2* gene and 5′-AGT GAC GAG TTT GTG GAC TCC TTT A for *Bad* gene. A scrambled sequence (sense – 5′-CGU ACU GUC GAC ACU GAA ACG GAdC dA; antisense – 5′-UAU CCG UUU CAG UGU CGA CAG UAC GdTdG) named si-NC, which was non-homologous to any human DNA sequence, was used as negative control. Cells were transfected with siRNA duplex using lipofectamine–RNAi MAX according to the manufacturer's instructions.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

The specific primers used to amplify the *Chk1*, *Chk2*, *Bad* and the house keeping gene *human porphobilinogen deaminase* (*hPBGD*) were as follows: *Chk1*, 5′-CCT TTG TGG AAG ACT GGG ACT and 5′-GAG GTT ATC CCT TTC ATC CAA C; *Chk2*, 5′-TCG TGA TGT CTC GGG AGT CG and 5′-GAG TTT GGC ATC GTG CTG GT; *Bad*, 5′-GCT CCG GCA AGC ATC ATC and 5′-CCA TCC CTT CGT CGT CCT C; *hPBGD*, 5′-TCT GGT AAC GGC AAT GCG G and 5′-GCA GAT GGC TCC GAT GGT G. *hPBGD* was amplified together with *Chk1* or *Chk2* or *Bad* in the same reaction, serving as an internal control.

2.7. Immunoflourescence staining

Cells were fixed with 4% paraformaldehyde, permeabilized and blocked in 1 × PBS containing 0.3% Triton X-100 and 5% normal goat serum, followed by incubation with monoclonal antibody against Bad (1:100) and then with goat-anti-mouse Alexa-488 conjugated secondary antibody (1:200). Nuclei were visualized by PI staining.

2.8. Analysis of mitochondrial membrane potential ($\Delta\Psi_m$)

Loss of $\Delta\Psi_m$ was determined using 5,5′,6,6′-tetrachloro- 1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1, Merck Calbiochem, Germany) [21]. Cells were washed three times with 1 × PBS and then incubated at 37 °C for 20 min with DMEM supplemented with 10% FBS and 10 μg/ml JC-1.

2.9. Statistical analysis

Values represent the mean ± S.D. from at least three independent experiments. Differences between the experimental groups were analyzed using Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Resistance to curcumin-induced apoptosis is closely related to G2/M arrest

To investigate whether G2/M arrest is associated with the resistance of cancer cells to curcumin-induced cytotoxicity, we first analyzed the apoptosis rate and the cell cycle distribution in hepatoma cell lines (Huh7, Hep3B, HepG2, SK-Hep-1 and QGY-7703) treated with 25 μM of curcumin for 24 h. Interestingly, the rate of apoptosis was inversely related to the percentage of cells at the G2/M phase after curcumin treatment (Fig. 1A–D and data not shown). Thereafter, the cell lines that are most sensitive (Huh7) and most resistant (Hep3B) to curcumin-induced apoptosis were chosen for further study in greater detail. We found that curcumin-induced apoptosis in Huh7 cells in a dose- and time-dependent manner (Fig. 1A), while Hep3B cells were very resistant to curcumin-induced apoptosis even at a high dose of 50 μM and an extended treatment time of 48 h (Fig. 1B). Furthermore, curcumin exposure did not significantly change the cell cycle distribution in Huh7 cells (Fig. 1C), but resulted in dramatic accumulation of Hep3B cells at the G2/M phase (Fig. 1D). These observations suggest that G2/M arrest may be associated with the resistance of Hep3B cells to curcumin-induced apoptosis. Therefore, we next examined whether G2 checkpoint was activated in curcumin-treated Hep3B cells.

3.2. Curcumin treatment results in activation of G2 checkpoint in Hep3B cells

Both DNA damage and environmental insult may activate the G2 checkpoint and result in G2/M arrest. It has been shown that DNA damage sensor Chk1/Chk2 plays a role in G2 checkpoint via ATM/ATR pathway [22], while the stress sensor p38 acts through ATM/ATR-independent pathway [23]. Activation of ATM/ATR-dependent and -independent pathways causes respective phosphorylation of Chk1-Ser317/Chk2-Thr68 and p38-Thr180/Tyr182, which consequently prevents the dephosphorylation of Thr14/Tyr15 in the Cdk1 kinase and in turn leads to G2/M arrest. To investigate whether G2 checkpoint is activated upon curcumin treatment, the kinetic changes of phosphorylated-Ser317-Chk1 (p-Chk1), phosphorylated-Thr68-Chk2 (p-Chk2), phosphorylated-Thr180/Tyr182-p38 (p-p38) and phosphorylated-Tyr15-Cdk1 (p-Cdk1) were analyzed. Considering that the proteins we analyzed might display cyclic expression during cell cycle, cells treated with DMSO (final concentration 0.1%, the vehicle of curcumin) were collected at each time point as those exposed to 25 μM curcumin and were used as negative control (Fig. 1E and F). Compared to DMSO-treated Hep3B cells, the p-Cdk1 level significantly increased in curcumin-treated cells, while total amount of Cdk1 remained the same between DMSO- and curcumin-exposed cells at every time point (Fig. 1F). The increased phosphorylation became evident at 12 h post-treatment, which is in accordance with the onset of G2/M arrest at this time point. Furthermore, the levels of p-Chk1 and p-p38 increased simultaneously as that of p-Cdk1 (Fig. 1F). However, no alteration in the amount of p-Chk2 was found (data not shown). As control, the total and p-Chk1, p-Chk2, p-p38 and p-Cdk1 proteins in DMSO- or curcumin-treated Huh7 cells were also analyzed. We found an dramatic increase of p-p38 at 12 h but no obvious change in the level of p-Chk1, p-Chk2 and p-Cdk1 in curcumin-exposed Huh7 cells compared with DMSO-treated ones (Fig. 1E). These results suggest that curcumin treatment results in G2 checkpoint activation in Hep3B cells via Chk1- or p38-Cdk1-mediated pathway, which may be associated with G2/M arrest and with the resistance of Hep3B to curcumin-induced apoptosis. Hep3B cells were thus employed to validate this contention in the following study.

3.3. Abrogation of Chk1-mediated G2/M arrest sensitizes curcumin-resistant Hep3B cells to apoptosis

To investigate whether Chk1 or p38 mediated the G2/M arrest in our cell model, we used RNAi technique to silence the expression of *Chk1* and *Chk2* genes (Fig. 2A) and found that knockdown of Chk1 eliminated the accumulation of p-Cdk1

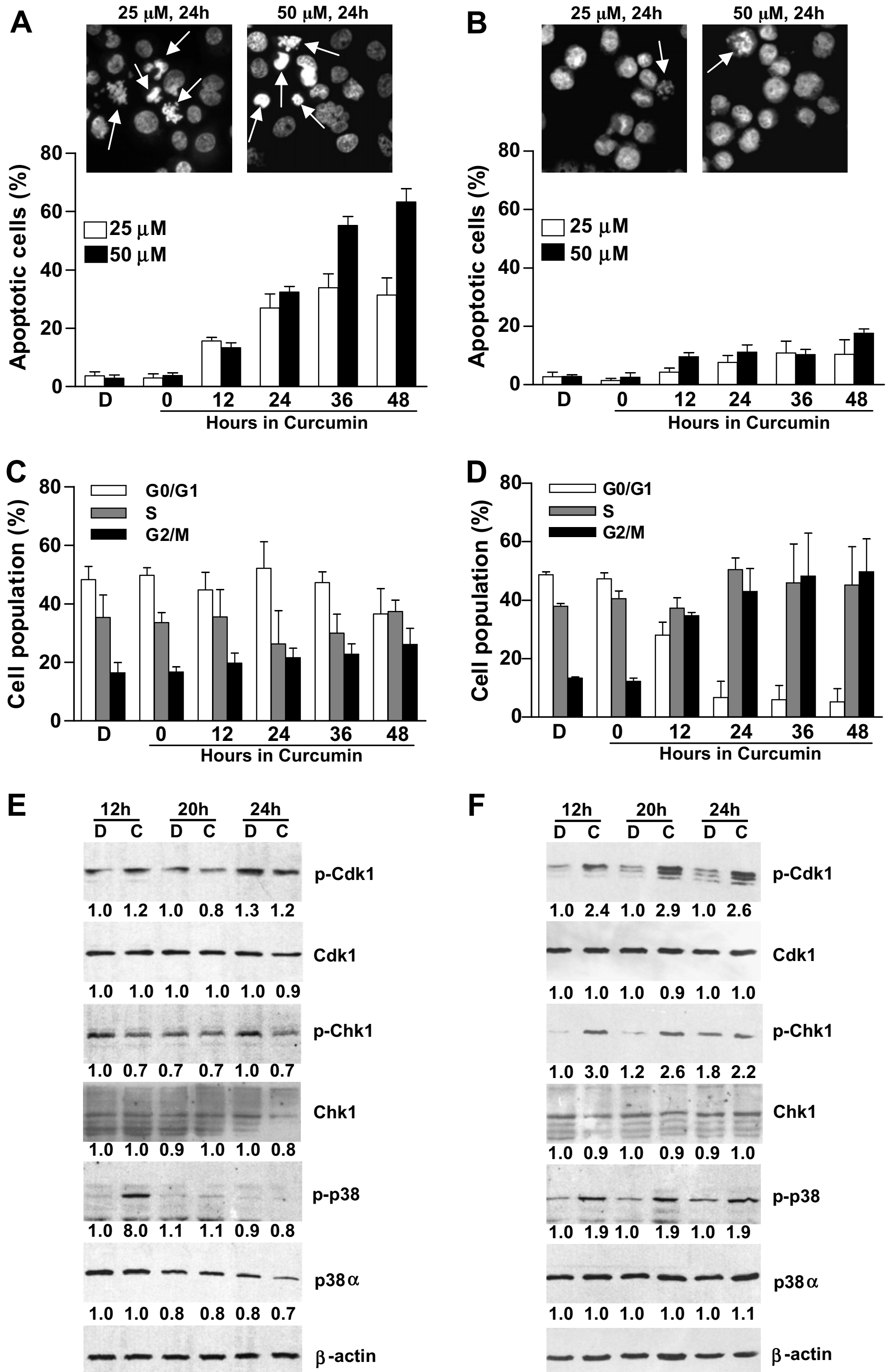

Fig. 1. Effect of curcumin on apoptosis rate, cell cycle distribution and expression of G2 checkpoint-related proteins. (A and B) The rate of apoptosis. Huh7 (A) or Hep3B (B) cells were treated with either DMSO for 48 h (vehicle control, indicated as D) or 25 or 50 μM curcumin for indicated times. Images presented over panels A and B show apoptotic nuclei (indicated by arrows) for Huh7 and Hep3B cells, respectively. (C and D) The cell cycle distribution. Huh7 (C) or Hep3B (D) cells were treated with DMSO (indicated as D) for 48 h or 25 μM curcumin for indicated times. (E and F) Expression of G2 checkpoint-related proteins. Huh7 (E) or Hep3B (F) cells were treated with DMSO (indicated as D) or 25 μM curcumin (indicated as C) for 12, 20 and 24 h. β-Actin was used as control for protein loading. The value under each band in (E) and (F) indicates the relative expression level of protein. The intensity for each band was densitometrically quantified and then normalized by the intensity of β-actin in each lane. The normalized intensity in the first lane was set as relative expression level of 1.

in curcumin-treated Hep3B cells (Fig. 2B, lanes 1, 2, 7 and 8). Meanwhile, Chk1 silencing dramatically attenuated curcumin-induced G2/M arrest (Fig. 2C) and conferred significant potentiation of apoptosis (Fig. 2D) in Hep3B cells. However, Chk2 knockdown failed to affect the level of p-Cdk1, to abrogate curcumin-induced G2/M arrest and to sensitize

Hep3B cells to apoptosis (Fig. 2B–D), which was consistent with our above observation that the level of p-Chk2 was not changed after curcumin treatment. Surprisingly, neither could double knockdown of the Chk1/Chk2 affect the rate of G2/M arrest and apoptosis in curcumin-treated cells, even the level of p-Cdk1 was obviously decreased (Fig. 2B–D).

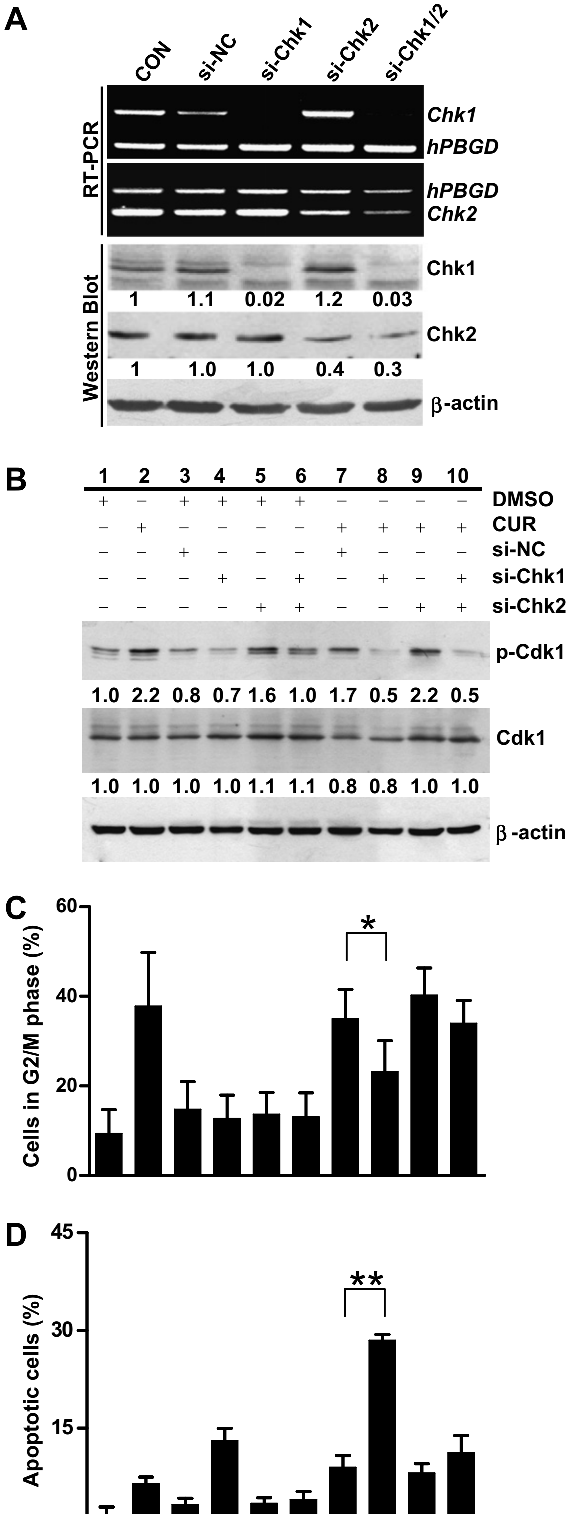

Fig. 2. Effect of Chk1 and Chk2 knockdown on curcumin-treated Hep3B cells. (A) The mRNA and protein levels of Chk1 and Chk2. Cells were analyzed 48 h after transfection with 50 nM of indicated siRNA by RT-PCR and Western blotting. The *hPBGD* and β-actin were served as internal controls for mRNA and protein levels, respectively. CON, Hep3B cells without any treatment; si-NC, a scrambled siRNA used as a negative control; si-Chk1, siRNA targeted Chk1; si-Chk2, siRNA targeted Chk2. (B) The level of Cdk1 phosphorylation. The value under each band in (A) and (B) indicates the relative expression level of protein. For details, see Fig. 1 legend. (C) The distribution of cell cycle. (D) The rate of apoptosis. Cells were transfected with 50 nM of indicated siRNA, followed by treatment with DMSO (as vehicle control) or with 25 μM curcumin for 24 h (for the analysis of Cdk1 phosphorylation and cell cycle distribution) or 48 h (for apoptosis analysis). The *X*-axis in panels B–D denoted the treatments for each sample, which were presented on the top of panel B. CUR, curcumin. $^{*}P < 0.05$; $^{**}P < 0.01$.

Next the role of p38 was characterized. Hep3B cells were exposed to 20 μM of p38 inhibitor (SB203580) for 2 h prior to curcumin treatment and were incubated with both SB203580 (20 μM) and curcumin (25 μM) for another 6, 12, 24 or 48 h. However, inhibition of p38 displayed no influence on the Cdk1 phosphorylation, G2/M arrest and apoptosis of curcumin-exposed cells (data not shown). These findings suggest that Chk1- but not p38-Cdk1-mediated G2/M arrest confers Hep3B cells with the resistance to curcumin-induced apoptosis and abrogation of this arrest sensitizes Hep3B to apoptosis.

3.4. Abrogation of G2/M arrest may sensitize curcumin-resistant Hep3B cells to apoptosis via upregulation of Bad and loss of $\Delta\Psi_m$

To investigate which apoptosis regulating pathways have been triggered when Chk1 is silenced, we first examined the mitochondrial pathway by determining the $\Delta\Psi_m$ with JC-1, a fluorescent dye that detects normal mitochondria as red color and depolarized mitochondria as yellow/green. Compared with the si-NC-transfected Hep3B cells, the si-Chk1-transfected Hep3B displayed an obvious loss of $\Delta\Psi_m$ after curcumin treatment (Fig. 3A).

It is well known that Bcl-2 family members play crucial roles in regulating the permealization of mitochondrial membrane [24], we thus assessed the relative expression levels of these proteins by Western blotting and immunofluorescent staining. The results showed that si-Chk1- but not si-NC-transfected cells presented an increase in the Bad protein level after curcumin exposure (Fig. 3B and C). However, the expression of other Bcl-2 family members including Bcl-2, Bcl-XL and Mcl-1 remained unchanged (Fig. 3B). To further investigate whether Bad is the critical molecule that triggered curcumin-induced apoptosis in Chk1-depleted-Hep3B cells, we employed RNAi technique to silence the expression of *Bad* gene and showed that Bad knockdown dramatically blocked the effect of Chk1 depletion in curcumin-treated Hep3B cells, in a dose-dependent manner (Fig. 4A). This observation was further validated on HCT-116($p53^{-/-}$), a colon cancer cell line with targeted deletion of p53. We found that HCT-116($p53^{-/-}$) cells, which displayed dramatic accumulation at the G2/M phase (from 18% to 70%) after curcumin treatment, were resistant to curcumin-induced apoptosis (Fig. 4B). Moreover, Chk1 silencing significantly sensitized curcumin-treated HCT-116($p53^{-/-}$) cells to apoptosis, while such Chk1-depletion-promoted apoptosis was attenuated by Bad knockdown (Fig. 4B). These

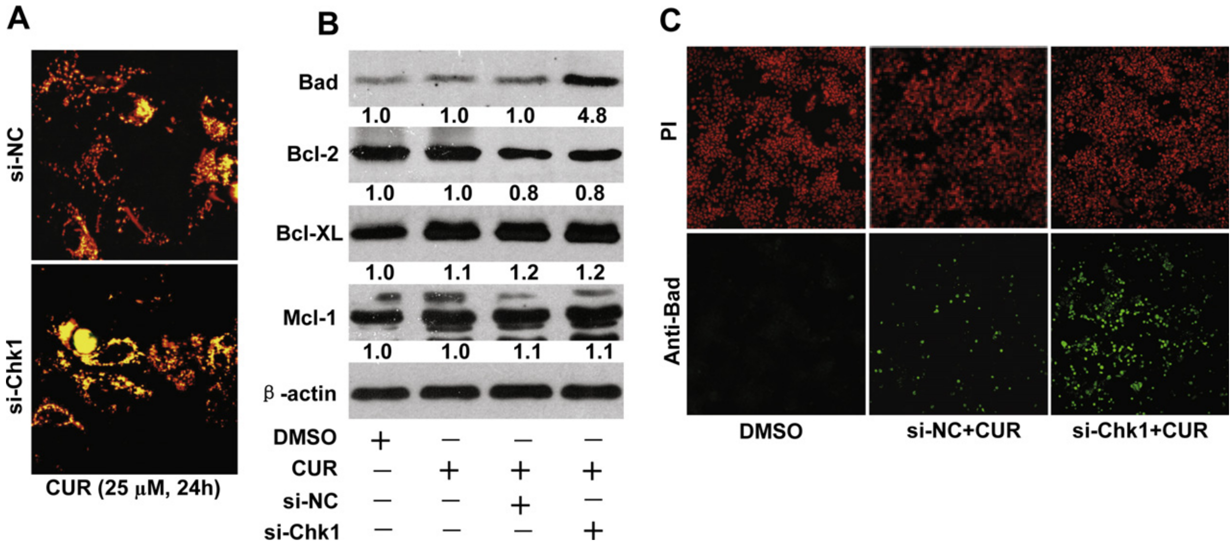

Fig. 3. Characterization of the signaling pathways triggered to induce apoptosis in curcumin-treated Hep3B cells after Chk1 depletion. (A) Analysis of mitochondrial membrane potential using JC-1. (B and C) The expression levels of Bcl-2 family members analyzed by Western blotting (B) or immunoflouresence staining (C). Cells were transfected with 50 nM of indicated siRNA for 48 h, followed by treatment with 25 μM curcumin for another 24 h and then applied to analysis. β-Actin was used as control for protein loading. The value under each band in (B) indicates the relative expression level of protein. For details, see Fig. 1 legend. CUR, curcumin; si-NC, a scrambled siRNA used as a negative control; si-Chk1, siRNA targeted Chk1; PI, propidium iodide.

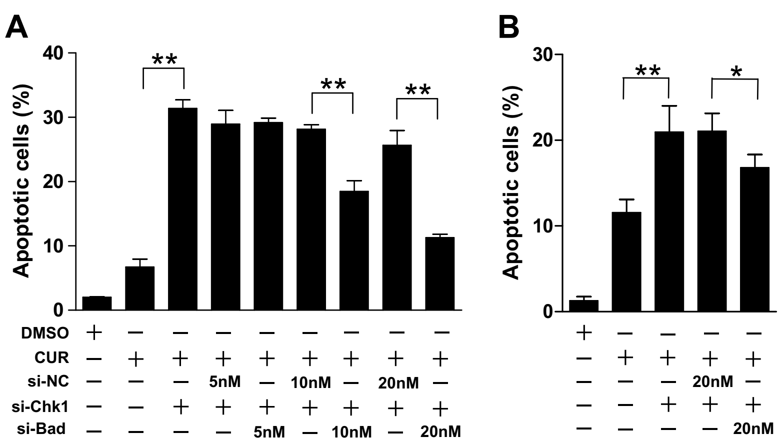

Fig. 4. Effect of Bad knockdown on curcumin-induced apoptosis in Chk1-depleted-Hep3B (A) and HCT-116($p53^{-/-}$) (B) cells. Cells were co-transfected with 50 nM of si-Chk1 and indicated concentration of si-Bad or si-NC for 48 h, followed by treatment with 25 μM curcumin for another 48 h. CUR, curcumin; si-NC, a scrambled siRNA used as a negative control; si-Chk1, siRNA targeted Chk1; si-Bad, siRNA targeted Bad. $^{*}P < 0.05$; $^{**}P < 0.01$.

results indicate that Chk1 inhibition sensitizes curcumin-resistant cells to apoptosis via upregulation of Bad and in turn the loss of $\Delta\Psi_m$.

4. Discussion

In the present study, we showed that Chk1-mediated activation of G2 checkpoint was required for curcumin-induced G2/M arrest, and Chk1 inhibition significantly abrogated this arrest and sensitized curcumin-resistant cells to apoptosis. Previous studies have also demonstrated that ablation of the G2 checkpoint could potentiate cell death induced by ionizing radiation [25], DNA-damaging agents [13,14] and non-DNA-damaging agents [26,27], strongly supporting G2 checkpoint as a potential therapeutic target for the reversal of drug resistance [15]. Undoubtedly, overcoming this resistance will significantly broaden the application of curcumin in cancer therapy. In addition, Hep3B cells have been shown to be resistant to different apoptotic inducing factors, such as FAS, TRAIL and paclitaxel [28–30]. It will be interesting to investigate whether G2 checkpoint is a target for reversing the resistance of Hep3B to other apoptosis stimuli.

Abrogation of curcumin-induced G2/M arrest and decreased phosphorylation of Cdk1 at the Tyr15 site were simultaneously found in Hep3B cells with single knockdown of Chk1. However, double knockdown of both Chk1 and Chk2 could not eliminate the G2/M arrest (Fig. 2C), although decreased accumulation of p-Cdk1 was observed (Fig. 2B, lane 10). One possible explanation for these observations is that Chk1 silencing may abrogate G2 checkpoint via Chk2-mediated signaling pathway. Aside from dephosphorylation at Tyr15, full activation of Cdk1 also requires binding to cyclin B1. It has been found that transcriptional activation of cyclin B1 by the forkhead box M1 (FoxM1) transcription factor is essential for timely mitotic entry [31], while FoxM1 protein is stabilized by Chk2 in response to DNA damage [32]. Therefore, we speculate that simultaneous dephosphorylation at Cdk1-Tyr15 (through Chk1 depletion) and increase in cyclin B1 expression (via Chk2-FoxM1 signaling pathway) are required for the checkpoint abrogation and cell cycle progression in our study model.

Increasing evidence indicates that ectopic expression of anti-apoptotic proteins such as Hsp70, Bcl-XL or Ku70 confers cancer cells with resistance to curcumin-induced apoptosis [33,34]. In addition, human colon cancer cells lacking Bax also resist curcumin-induced apoptosis [35]. Interestingly, we found that expression of Bad, the proapoptotic molecule in the Bcl-2 family, increased obviously after release of G2/M arrest (Fig. 3B and C). Moreover, Bad knockdown dramatically attenuated curcumin-induced apoptosis in Chk1 depleted-cells (Fig. 4). These results indicate the existence of crosstalk between the signaling pathways that control apoptosis and the cell cycle, when curcumin-induced G2/M arrest is released by Chk1 knockdown.

In summary, we characterize the molecular mechanisms underlying curcumin resistance as well as the reversal of this resistance. Our data indicate that (1) Chk1-mediated G2/M arrest may serve as a mechanism for the resistance of cancer cells to curcumin-induced apoptosis; (2) Chk1 represents a potential target for the reversal of curcumin resistance; (3) Bad is the critical molecule that triggered apoptosis in curcumin-resistant cells after Chk1 depletion. These findings should be helpful for the clinical application of curcumin.

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